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癌関連抗原に対するモノクローナル抗体の核医学的応用

細野 真

Human/mouse chimeric antibodies show low reactivity with human anti-murine antibodies (HAMA)

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Construction of Immunoradiometric Assay for Circulating c-erbB-2
Protooncogene Product in Advanced Breast Cancer Patients

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Running Title: Serum c-erbB-2 product levels

Key Words: tumor marker, c-erbB-2 product, circulating antigen, immunoradiometric assay, breast cancer

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SUMMARY

The human c-erbB-2 protooncogene product (erbB-2 protein) is a 185 kilodalton glycoprotein closely related to epidermal growth factor receptor protein. In this study, we measured the concentration of circulating erbB-2 protein in cancer patients by means of a new immunoradiometric assay (IRMA). Two monoclonal antibodies (MoAbs), SV2-61γ and 6G10, recognize erbB-2 protein but bind to separate epitopes. SV2-61γ was used as an immunoabsorbent and 6G10 as an ¹²⁵I-labeled probe. A serum was considered positive for erbB-2 protein if the percent binding exceeded the mean of the normal group by more than 3 standard deviations. Eleven of 21 patients with advanced breast cancer and 1 of 15 with advanced gastric cancer were positive. Serum erbB-2 protein levels correlated well with the therapy and the status of the patients with breast cancer. On the contrary, all patients with advanced colon, ovarian, or pancreatic cancers, showed levels below the cutoff value. These results suggest that circulating erbB-2 protein can be measured using the newly constructed IRMA. Since c-erbB-2 protooncogene amplification and overexpression are accepted as a good marker of aggressiveness, relapsing potency, and poor prognosis, this IRMA should be a promising tool with which to help manage breast cancer patients.

INTRODUCTION

The *c-erbB-2* protooncogene encodes a protein that is similar to the epidermal growth factor (EGF) receptor but is distinct from it.¹⁾

Recently we reported that the *c-erbB-2* protooncogene product (*erbB-2* protein), overexpressed on the surface membranes of various adenocarcinomas, is a useful target in immunoscintigraphy using monoclonal antibodies (MoAbs).²⁾ This protein is reportedly expressed in various adenocarcinomas including breast, gastric, colon cancers,³⁾ whereas EGF receptors are expressed on squamous cell carcinomas and gliomas as well as adenocarcinomas.⁴⁻⁶⁾ Amplification and overexpression of the *c-erbB-2* gene correlate with tumor aggressiveness and unfavorable clinical outcome, especially in patients with breast or gastric cancer.⁷⁻¹¹⁾ In addition, this protein is detectable in the sera of breast cancer patients.^{3,12)} In this study, we constructed an immunoradiometric assay (IRMA) to determine whether circulating *erbB-2* protein is detectable in the sera of patients with breast, gastric, colon, ovarian and pancreatic cancers.

MATERIALS AND METHODS

Monoclonal Antibodies

MoAbs SV2-61γ and 6G10 (Nichirei, Tokyo, Japan) recognize the extracellular domains of *erbB-2* protein, and bind separate epitopes. SV2-61γ (IgG1) was generated by immunizing mice with SV11 cells in which the *c-erbB-2* complementary DNA was expressed.¹³⁾ The MoAb 6G10 (IgG2a) was obtained by immunizing mice with recombinant *c-erbB-2* gene transfected A4 cells.³⁾

Standard antigens

Standard antigens were obtained by solubilizing the cell membrane of A4 (*c-erbB-2* transfected NIH3T3) cells cultured in Eagle's minimum essential medium (Nissui Pharmaceutical Co., Tokyo) supplemented with 10% fetal calf serum and 0.03% L-glutamine. The cells were transplanted subcutaneously to a BALB/c-nu/nu mouse, then 2 weeks later, the A4 xenograft was removed. Cells were obtained by passing tumor specimens through a stainless steel mesh and were suspended in phosphate buffered saline (PBS). After adding 4ml 0.5% Triton X-100 in PBS, 1×10^8 cells were incubated on ice for 1 h. Nuclei and debris were removed by centrifugation ($10000 \times g$, 10 min). The supernatant was serially diluted with dilution buffer (Daiichi Radioisotope Laboratories Ltd., Tokyo, Japan) and the antigen level of the supernatant diluted to 1/8 was defined as 500U/ml arbitrarily.

Preparation of beads

Polystyrene beads (7mm in diameter) were coated with MoAb SV2-61 γ in 1mM phosphate buffer, pH6.4 containing 5% MoAb at 4°C for 20 h. After washing the beads three times with distilled water, non-specific binding was blocked by processing in 50mM PBS containing 0.5% bovine serum albumin (BSA).

Radio-iodination of monoclonal antibody

MoAb 6G10 was radio-iodinated using the chloramine-T method. Purified antibody (20 μ g) in 0.3M phosphate buffer, pH7.5 and 125 I (0.4mCi) for protein labeling (Amersham International plc, Buckinghamshire, UK) was mixed with 2.5 μ g of chloramine-T (Nakarai Chemicals, Kyoto, Japan). After a 5 min incubation, 125 I-labeled MoAb was separated from free 125 I through a Sephadex G-25 gel. The specific activity was 444MBq(12mCi)/mg.

Sera

Sera were obtained between 1989 and 1990 in Kyoto University Hospital from patients with breast cancer(n=21), ovarian cancer(n=11), colon cancer(n=15), gastric cancer(n=15), and pancreatic cancer(n=10). Serum samples were stored at -20°C until use. All cancer patients underwent treatment in Kyoto University Hospital, and diagnoses were based upon surgical and/or histological findings. Clinical examinations, laboratory tests, and radiological findings demonstrated that all of them were in Stage IV or the recurrent stage, at the time of serum sampling. Sera were also taken from 20 normal adults; 10 males and 10 females.

Immunoradiometric assay(IRMA)

An IRMA was constructed using SV2-61 γ as the immunoabsorbent and 6G10 as the 125 I-labeled probe. Radio-iodinated 6G10 was diluted with tracer dilution buffer (Daiichi Radioisotope Laboratories, Ltd., Tokyo, Japan) at a specific activity of 60000cpm/100 μ l. Fifty microliters of standard antigens or serum samples were incubated with 100 μ l of 125 I-labeled 6G10 and SV2-61 γ -coated beads for 6 h at room temperature. After washing the beads three times with distilled water, the radioactivity bound to the beads were measured using a well-gamma counter. Bound radioactivity was converted to erbB-2 protein U/ml by reference to a standard curve.

CA15-3 assay

In all the patients with breast cancer, the serum CA15-3 (14,15) levels were determined using a commercially available kit (Centocor, Malvern, PA, USA) with a cutoff value of 28U/ml. The procedure of the assay was as described by the manufacturer. CA15-3 levels were compared with those of erbB-2 protein.

RESULTS

The standard curve of the assay is shown in Fig. 1. The prozone phenomenon was observed with the less diluted supernatant of the standard antigens. Two of the serum samples

were diluted with dilution buffer in the geometrical series, and the erbB-2 protein levels of diluted samples are illustrated in Fig. 2. Both of the plots showed good linearity from 1 to 1/32 dilution. In the 20 healthy controls, the erbB-2 protein concentrations ranged from 0 to 2.6U/ml. The mean and the standard deviation was 0.4 and 0.7, respectively, and there was no difference between males and females. The cutoff value was 3 U/ml, determined as a mean plus 3 standard deviations. All the healthy controls showed levels below the cutoff value (Fig. 3). Twelve of 21 patients (57.1%) with advanced breast cancer were positive. On the contrary, all 15 patients with colon cancer, all 11 patients with ovarian cancer, all 10 patients with pancreatic cancer, and 14 of 15 patients with gastric cancer had erbB-2 protein levels below the cutoff value of 3U/ml.

Fifteen of 21 patients (71.4%) had positive CA15-3 levels with a cutoff level of 28U/ml. The patients were divided into four groups on the basis of erbB-2 protein and CA15-3 levels (Table I). The correlation coefficient between erbB-2 protein and CA15-3 was 0.425.

Serum samples were serially taken from 2 patients (A and B) with breast cancer, and the changes of serum erbB-2 protein levels and CA15-3 levels were determined. Patient A, a 53-year-old female, who had undergone left mastectomy 2 years previously, had multiple liver metastases, bone metastases, lung

lymphangitis carcinomatosa and ascites. Liver metastases were treated by transcatheter arterial chemoinfusion and embolization, after which, the serum erbB-2 protein level decreased. However, the erbB-2 protein levels increased again in accordance with the extension of metastatic lesions. She expired 11 months after the diagnosis of recurrence (Fig. 4). Patient B, a 52-year-old female with left breast cancer, had multiple bone, bone marrow and brain metastases at the time of the first consultation, and mastectomy was not performed. During admission, she was treated by chemotherapy using fluorouracil, cyclophosphamide and tamoxifen citrate. Metastatic lesions improved and the serum erbB-2 protein level decreased (Fig. 5).

In both patients, serum erbB-2 protein levels determined by our IRMA changed in parallel with serum CA15-3 in response to the therapy and the status of patients.

DISCUSSION

The protooncogene *c-erbB-2* encodes a 185 kilodalton transmembrane tyrosine kinase, which is similar to epidermal growth factor (EGF) receptor, although EGF does not bind to nor activate erbB-2 protein. Recently heregulin (HRG) proteins interacting specifically with erbB-2 protein have been identified as the probable natural ligands for erbB-2 protein.¹⁶⁾

Overexpression of the erbB-2 protein, generally as a consequence of gene amplification, has been reported in human breast, gastric, ovarian, colon, pancreatic, and urinary bladder

cancers. Some clinical studies have disclosed association of c-erbB-2 gene amplification and aggressiveness, relapsing potency and poor prognosis of these cancers. 7-11,17-20)

In this study, we detected and quantified erbB-2 protein in sera from patients with adenocarcinomas. Patients with breast cancer had high concentrations and positive rates compared with other adenocarcinoma patients. According to some immunohistochemical reports, about 30% of breast cancer patients had erbB-2 protein.^{21,22)} An immunohistochemical study has shown that 28(14.3%) of 196 advanced gastric cancer cases were positively stained for erbB-2 protein.¹¹⁾ In our study, 57.1 and 6.7% of breast and gastric cancer patients, respectively, had positive serum erbB-2 protein. Our breast cancer patients demonstrated a higher positive rate than those studied immunohistochemically and reported elsewhere. This may be because all our patients were in the advanced stages. Yu et al. reported 45.7% of the postoperative recurrent breast cancer patients were positive serum erbB-2 protein.¹²⁾ Their positive rate may be considered equivalent to that of our advanced cancer patients.

CA15-3, a tumor marker detectable by two monoclonal antibodies 115D8 and DF3, has been used for the diagnosis and follow-up of breast cancer patients. The positive rate of CA15-3 in advanced stages is reported to be 70%.^{23,24)} In our study, 57.1% of advanced breast cancer patients had positive serum erbB-2 protein levels, while 71.4% of them showed positive CA15-3. Furthermore, erbB-2 protein represented only a weak correlation with CA15-3. One patient showed positive erbB-2

protein and negative CA15-3, and 4 patients were with negative erbB-2 protein and positive CA15-3. Thus, erbB-2 protein is not only biochemically a completely distinct substance from the antigen determined with CA15-3 assay, but it may be considered as a different tumor marker from CA15-3 in monitoring breast cancer patients. Further study with more patients must be undertaken to estimate the usefulness of this erbB-2 protein assay in comparison with CA15-3.

Gene amplification and overexpression of the c-erbB-2 have clinically prognostic significance for patients with c-erbB-2 positive tumors. Thus, gene analyzing and immunohistochemical tests are essential in defining an individual prognosis. However, these tests need tumor specimens, cost a lot of time and labor, and cannot deal with many samples in a short time. Compared with them, IRMA, a highly simpler method, has several advantages; patient's serum can be obtained easily and repeatedly, many samples are measured at the same time, and IRMA can quantify them numerically. In addition, our construction of IRMA measuring oncogene product suggests that other oncogene products can be determined with IRMA, and such serodiagnoses may supplement or replace the gene analyzing and immunohistochemical tests in some clinical practices. Although additional studies, including patients in the earlier stages, will be necessary to evaluate the predictive value of serum erbB-2 protein, our IRMA for erbB-2 protein seems to be a useful tool with which to monitor breast cancer patients.

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Legends

- Fig.1. Standard curve. Bars indicate the standard deviations of five different assays. cpm: radioactivity of the beads.
- Fig.2. Two samples of erbB-2 protein positive serum were diluted and measured by the IRMA.
- Fig.3. Serum c-erbB-2 product levels in healthy controls and adenocarcinoma patients. One large ● indicates ten patients.
- Fig.4. Patient A. 53 year-old female with breast cancer showing liver, lung, and bone metastases. TAE: transcatheteral arterial embolization of metastatic liver lesions. Serum erbB-2 protein levels(●); CA15-3 levels(O).
- Fig.5. Patient B. 52 year-old female breast cancer patients with bone and bone marrow metastases. Chemotherapy continued for 3 months in hospital. Serum erbB-2 protein levels(●); CA15-3 levels(O).

Table I. Relationship between erbB-2 Protein and CA15-3 Levels in the 21 Advanced Breast Cancer Patients

		No. of patients (%)	
		CA15-3	
		Positive	Negative
erbB-2 protein	Positive	11 (52.4%)	1 (4.8%)
	Negative	4 (19.0%)	5 (23.8%)

Fig. 1

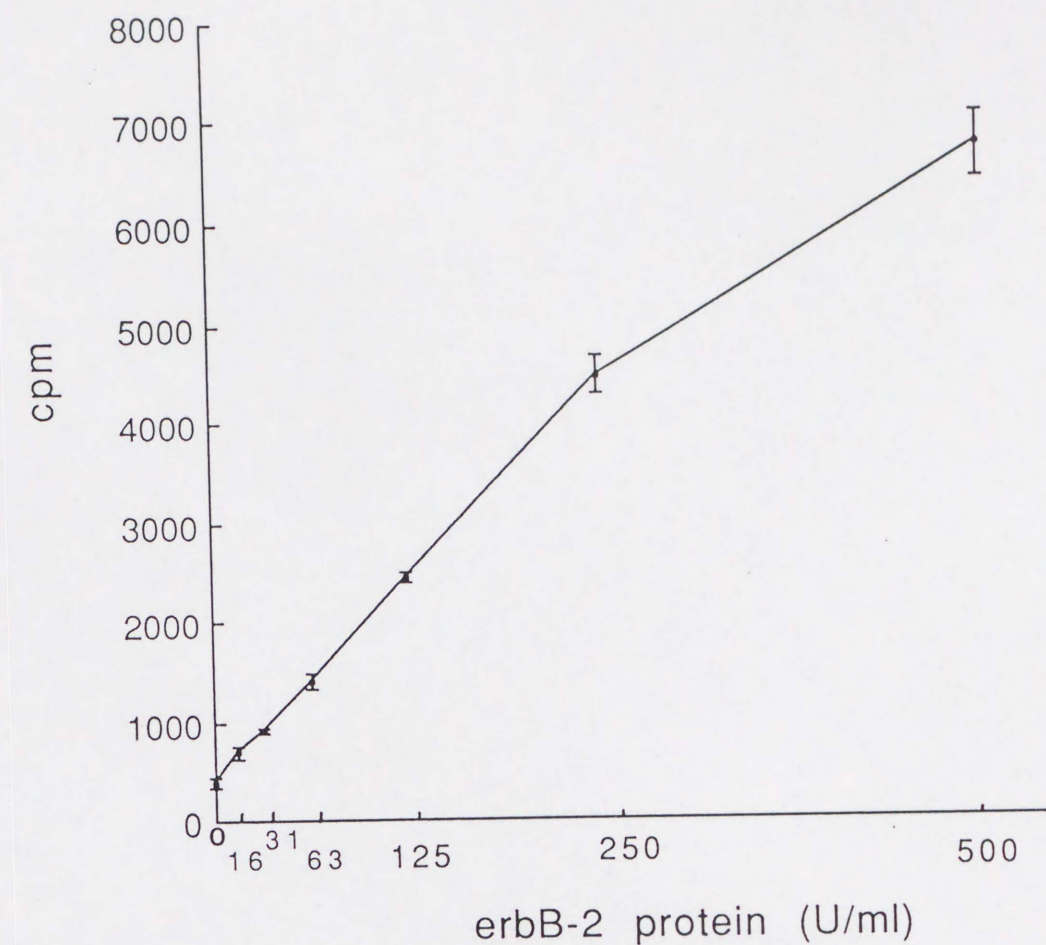


Fig. 2

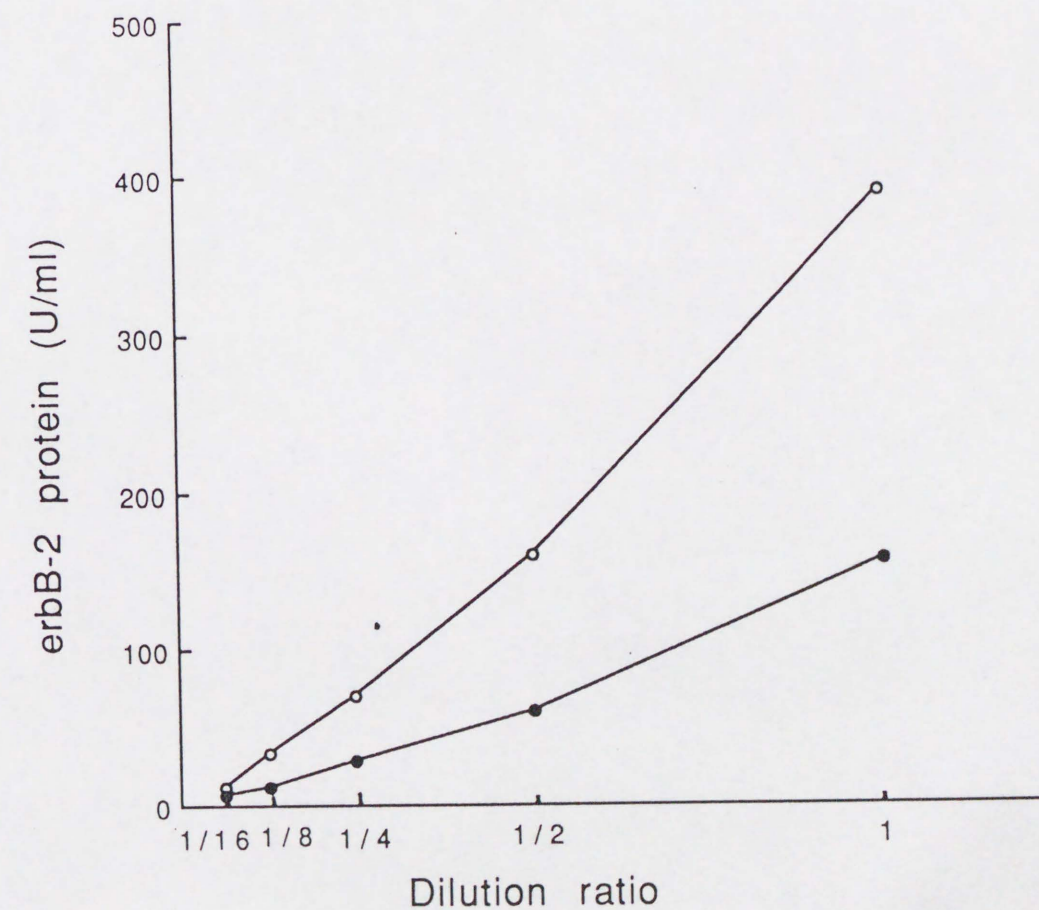


Fig. 3

Diseases	n	Positive rate (%)	erbB-2 product levels (U/ml)				
			3	10	100	1000	
Healthy controls	20	0	●●				
Breast cancer	21	57.1	●●●●	●●●●●	●●	●	●
Ovarian cancer	11	0	●●				
Colon cancer	15	0	●●●				
Gastric cancer	15	6.7	●●●	●			
Pancreatic cancer	10	0	●				

Fig. 4

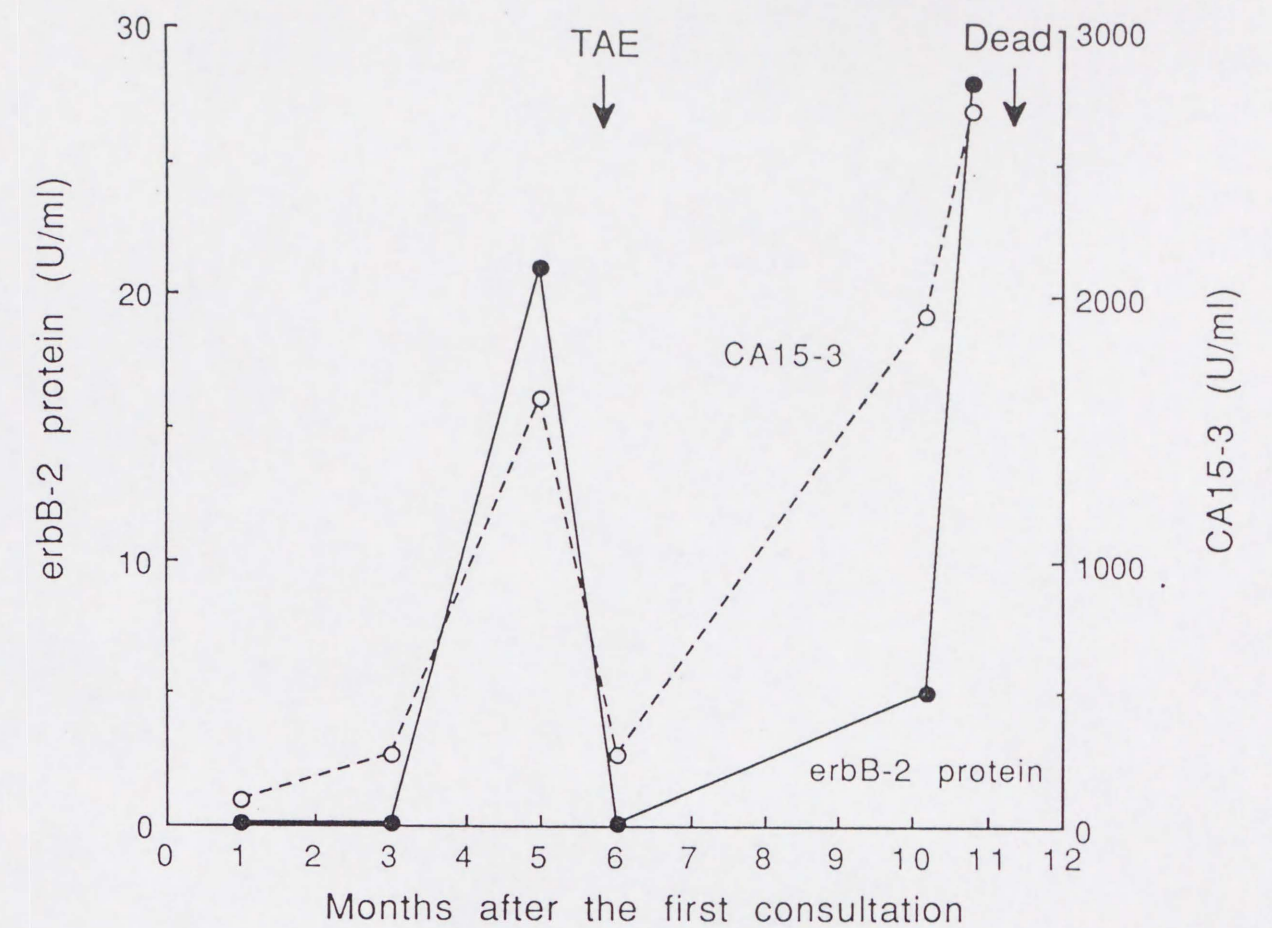


Fig. 5

